# Twenty Years of PSA: From Prostate Antigen to Tumor Marker

Gabriela De Angelis, MD, PhD,* Harry G. Rittenhouse, PhD,† Stephen D. Mikolajczyk, BA,‡ L. Blair Shamel, MS,§ Axel Semjonow, MD, PhD‖

*Institute for Clinical Chemistry and Laboratory Medicine, Münster, Germany; †Gen-Probe, Incorporated, San Diego, CA; ‡Biocept Inc., San Diego, CA; §DiagnoCure, Incorporated, St. Foy, Quebec, Canada; ‖Prostate Center, Department of Urology, University Clinic, Münster, Germany

*The measurement of prostate-specific antigen in serum is credited with dramatic advances in the early detection of men with prostatic carcinoma. This report summarizes the history of biochemical research and the current understanding and application of prostate-specific antigen in prostate cancer diagnostics.*
[Rev Urol. *2007;9(3):113-123*]



**Key words:** Prostate cancer • Prostate-specific antigen • Human kallikrein • Immunoassays

In the 1960s, antigens with properties of prostate organ specificity were characterized in prostate-tissue extracts.[1,2] Independently between 1966 and 1971, Hara and colleagues in Japan identified a prostate-specific protein in seminal fluid—"gamma seminoprotein."[3,4] An assay for gamma seminoprotein was subsequently developed for forensic use in rape cases. In 1978, Sensabaugh purified a prostate-specific protein from seminal fluid, p30, which was also used for forensic purposes.[5]

Following discovery of prostate antigen in prostate cancer tissue and sera by the working group headed by Wang at Roswell Park Cancer Institute, the question of whether gamma seminoprotein p30 and a protein with similar properties, E1 antigen,[6,7] represent the same protein as prostate antigen became the focus of numerous studies.[5,8,10-18]

In 1979, for the first time, the Wang group reported the immunoprecipitation of an antigen from a pool of normal, hyperplastic, and malignant prostate tissue.[8] They were able to show that this antigen was clearly prostate localized (ie, it was not detected in bones, kidney, intestines, liver, or spleen). It was also shown that it differed immunologically and chemically from prostatic acid phosphatase (PAP), which had been used since 1938 for diagnosis of prostate cancer.[9]

In 1992 it was determined that all of these proteins had identical amino acid sequences and, therefore, were encoded by the same gene.[19] Now it is recognized that gamma seminoprotein p30 and E1 are the same protein as prostatic antigen (PA), identified by Chu's laboratory in prostate cancer patients and subsequently known as prostate-specific antigen (PSA).[19]

## Biochemistry and Physiology of PSA

Sensabaugh's method for isolating p30 from seminal plasma[5,16] became established as a standard method for preparation of purified PSA. There was a great deal of interest and much initial difficulty in determining the biochemical and physiological role for this protein. In 1984, Chu's laboratory reported that PSA functioned as a protease, although they concluded that it was not a serine protease.[20] A year later, the first reported physiological role for PSA, the proteolytic cleavage of seminal vesicle proteins, was shown to occur primarily by cleavage at basic amino acids characteristic of trypsin-like serine proteases.[21] By analysis of the amino acid sequence in 1986, Watt and colleagues recognized that PSA is structurally similar to serine proteases and kallikreins and showed experimentally that it had both trypsin and chymotrypsin-like enzymatic activities.[18] Finally, Akiyama and associates demonstrated conclusively in 1987 that the true enzymatic activity of this protein was that of a chymotrypsin-like serine protease, with high selectivity toward only certain hydrophobic amino acids.[22]

It was difficult to establish the true enzymatic properties of PSA because it is a very poor protease, with low enzymatic activity. By comparison, human glandular kallikrein 2 (hK2), a similar protease that is coexpressed with PSA in the prostate, has a trypsin-like catalytic activity 20,000 times higher than the chymotrypsin-like activity of PSA.[23,24] Therefore, contamination of PSA with less than 1% of hK2 or a similar protease would produce an apparent trypsin-like activity higher than the PSA chymotrypsin-like activity.

In 1988, Lilja revealed the probable physiological function of PSA, and this remains the most commonly accepted hypothesis.[25] In the seminal fluid, PSA cleaves the gel-forming proteins from the seminal vesicles (seminogelins I and II and fibronectin) initiating liquefaction of the ejaculate, thereby increasing the motility of sperm cells and aiding fertilization. The physiological reason for high concentrations (typically 1-2 mg/mL) of a low-activity protease in the seminal plasma is unknown. It was shown, however, that hK2 was also active in seminal plasma and cleaved the seminogelins and fibronectin at different amino acid sites than PSA.[26]

*PSA is a very poor protease, with low enzymatic activity.*

## PSA and the Homologous Prostate Kallikrein, hK2

"Kallikreas" is the Greek word for the pancreas, which Kraut and colleagues[27] assumed to be the primary source of the hypotensive factor that they called human kallikrein 1 (hK1). We know now that hK1 is also expressed in the salivary glands and kidney and has powerful pain-producing and vasodilation effects through the enzymatic release of kinins. The ability to release kinins is viewed by some as the definition of the true kallikrein, and this function has been extensively investigated.[28]

PSA was the second member assigned to the human kallikrein family because of its structural homology with hK1, even though it had an entirely different function. In the late 1980s a third human kallikrein was discovered, hK2, Like PSA, hK2 is prostate localized.[29] Formal nomenclature was established for kallikreins, and PSA was named hK3.[30]

PSA and hK2 are closely related in terms of molecular structure, serine protease activity, and prostate compartmentalization,[31] but hK2 has a trypsin-like specificity restricted to arginine residues[23,32] and is a much more potent enzyme, with perhaps more than 20,000-fold greater enzymatic activity toward specific chromogenic peptide substrates.[24,33] The very high levels of PSA in seminal plasma may compensate for its relatively low enzymatic activity. In general, hK2 is present at only 1% to 2% the amount of PSA in prostate, seminal plasma, and serum.[26,34-36] hK2 was shown to autoactivate in vitro by cleavage at a propeptide arginine residue and release of mature hK2.[24] PSA contains the same propeptide arginine residue and does not autoactivate. Together with the high relative enzymatic activity of hK2 and prostate colocalization with PSA, this suggested that hK2 might regulate the activity of PSA.

In 1997, Takayama,[37] Kumar,[38] Lövgren,[39] and their colleagues independently produced the PSA precursor (proPSA) in recombinant form and reported its activation by hK2 in vitro. They found that hK2 cleaves the short 7-amino acid, N-terminal propeptide of the 244-amino acid precursor form of PSA, resulting in development of the mature active PSA molecule with 237 amino acids.

PSA has an 80% amino acid sequence identity with hK2.[40] This close homology led to speculation that immunoassays for PSA may cross-react significantly with hK2. The relatively low amounts of hK2 compared with PSA probably would not change serum PSA levels significantly in most cases, but a few specimens with relatively high hK2 levels may be affected. It was demonstrated in 1999 that at least some monoclonal antibody PSA assays do not cross react with pure hK2.[41] Polyclonal antibodies to PSA,

however, do cross react with hK2 and vice versa with polyclonal antibodies to hK2.

In general, current monoclonal PSA immunoassays would not be expected to be influenced by serum hK2, but the functional and enzymatic studies of PSA may be affected significantly by the much higher enzyme activity of hK2 relative to PSA. In fact, the earliest physiological study showing that PSA cleaved the seminogelin proteins in seminal plasma showed cleavage at arginine and lysine residues and attributed a trypsin-like activity to the preparations of purified PSA.[21] Even when the structural rationale and the demonstrated chymotrypsin-like activity for PSA were clarified,[18,22] virtually all of the published reports of PSA enzymatic activity throughout the 1990s continued to observe at least some trypsin-like activity.

Frenette and associates reported in 1998 that highly purified PSA was still contaminated with very low amounts of hK2.[42] It was not until recombinant PSA was purified from nonphysiological sources that a truly hK2-free preparation of PSA was demonstrated.[43] The quantity of contaminating hK2 would not be of consequence for PSA standardization methods, but contamination with hK2 and possibly other trypsin-like kallikreins now known to be in seminal plasma[44] will continue to be an issue because of the low enzymatic activity of PSA. Similar concerns are evident in studies showing the PSA inactivation of insulin-like growth factor binding protein-3.[45]

## PSA and the Human Kallikrein Family of Proteins

Until the early 1990s, the human kallikrein family was thought to consist only of hK1, hK2, and PSA (hK3).[46] The paucity of human kallikreins was considered an anomaly because other mammals, including rats and mice, were known to contain 20 or more kallikrein genes.[47] In the late 1990s, 12 new human kallikrein genes were discovered, several in the Diamandis laboratory.[48] All of the 15 human kallikrein genes are clustered in a single gene locus 19q13.4.[48,49] The entire human kallikrein family may consist of these 15 genes, because they are contiguous and bordered by nonkallikrein genes on both the centromere and telomere sides of the chromosome. Active research is in progress in order to understand the function and, in some cases, potential diagnostic or therapeutic roles of these homologous proteins.[48]

Only hK2, PSA (hK3), and hK4 appear to be primarily prostate localized, and it is tempting to speculate that these 3 homologous serine proteases interact in some way physiologically within the prostate. Because hK4 appears to be similar to hk2, it could serve some of the same putative functions ascribed to hK2, such as the activation of proPSA[50] (Figure 1).

*Contamination with hK2 and possibly other trypsin-like kallikreins now known to be in seminal plasma will continue to be an issue.*

## PSA and the Detection of Prostate Cancer

In 1980, Papsidero and colleagues[51] at Roswell Park found that PSA occurs in the serum of patients with prostate cancer and not only in prostate tissue and seminal plasma. Using an insensitive immunoelectrophoresis method that detected a minimum of 500 ng/mL PSA (compared with $< 0.01$ ng/mL for modern immunoassay techniques), sera from 219 patients with advanced prostate cancer and 175 patients with other types of cancer were tested. Using this technique, PSA was detected in the serum in only 17 of the 219 patients with prostate cancer and attributed to the pathological condition of the prostate, by analogy to PAP.[51]

In 1980, Kuriyama and associates, working in the same group, reported the first sensitive PSA immunoassay.[52,53] PSA could be quantified more accurately with this assay, and the clinical sensitivity for prostate cancer was much higher. It was soon discovered that elevated levels of PSA often occurred as well in non-cancer patients with enlarged prostate glands and clinical benign prostatic hyperplasia.[54] The lack of specificity of PSA for prostate cancer significantly delayed clinical application. This problem was amplified by the skepticism of clinicians toward prostate cancer tumor markers after years of overpromotion of the prostatic acid phosphatase assay as a "Male PAP test." Only when high-quality clinical studies validated the potential of PSA did it become an important serum test for the management, and later the detection, of prostate cancer.

Another key to the adoption of PSA was the concurrent improvement in therapies for prostate cancer. The development of the anatomic, nerve-sparing prostatectomy by Walsh at Johns Hopkins Medical Institute, Baltimore, MD, in the early 1980s made surgery for the disease more effective with lower morbidity for the early disease detected by PSA tests.[55] Conversely, the ability of PSA to detect very early signs of relapse has allowed development of more effective therapies for metastatic disease.

## Free and Complexed PSA in Serum

In 1987, molecular sieve methods unexpectedly found 2 forms of PSA

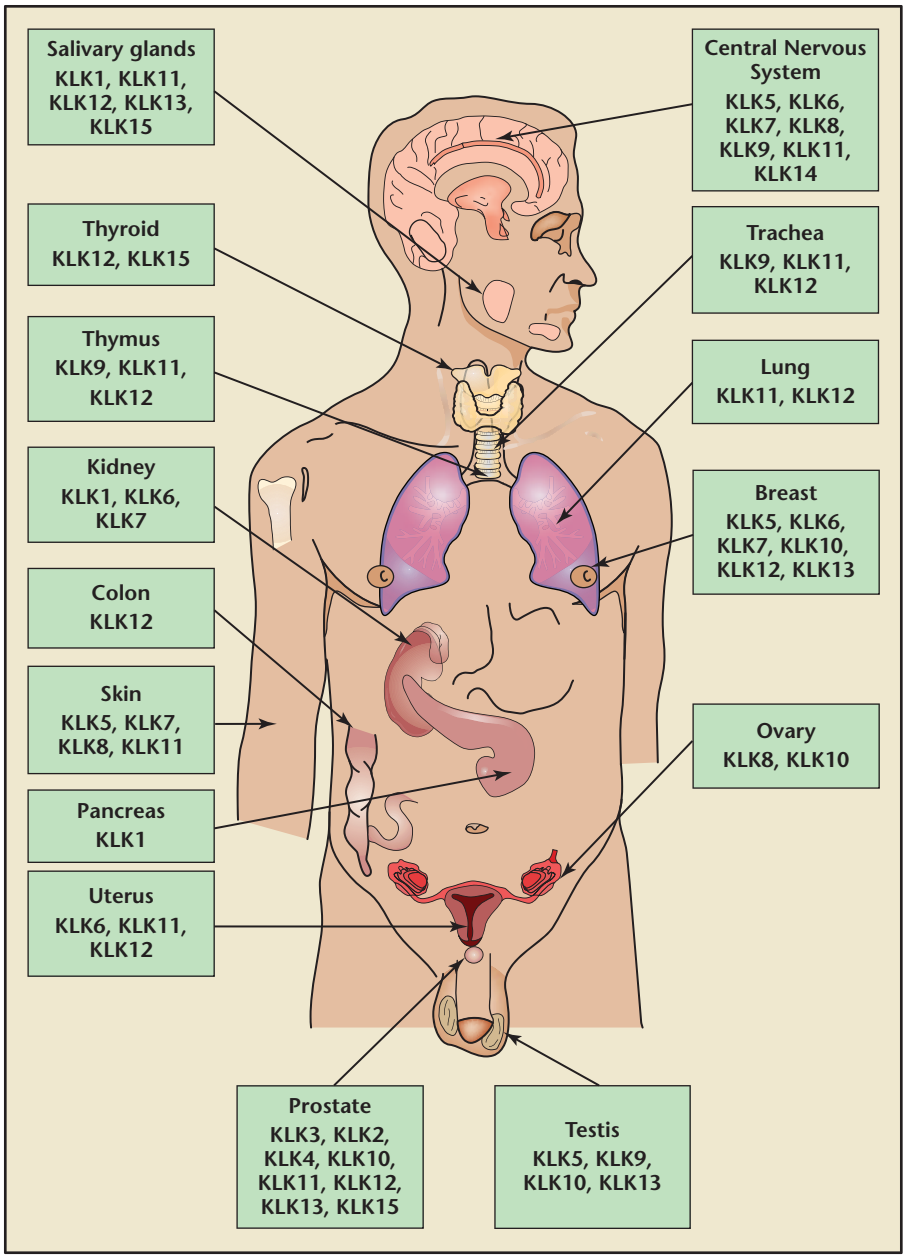


Figure 1. *Schematic representation of major expressions of human kallikreins in various tissues. Adapted from Yousef and Diamandis.*[48]

in the sera of patients with prostate cancer: a small (36 kDa) and a large (90-100 kDa) molecular form. The expected molecular size of PSA from biochemical characterization of tissue PSA by amino acid sequencing is about 26 kDa. The authors attributed the larger molecular PSA form, 90-100 kDa, to polymerization or possibly the binding of PSA autoantibodies to PSA.[14] Many years later the free and complex forms of PSA were discovered in the patient serum, accounting for the 2 forms mentioned.

It was Lilja and Stenman in the early 1990s who first discovered the identity of the 2 major molecular forms of PSA in serum.[56,57] PSA exists in serum predominantly as a complex with the protease inhibitor alpha-1-antichymotrypsin (ACT), whereas only about 10% to 30% is present as uncomplexed or free PSA. Circulating antiproteases, such as ACT and alpha-2-macroglobulin (α-2-MG), protect against active proteases. For example, the trypsin-like protease hK1 is complexed primarily with α-2-MG and α-1-protease inhibitor. Anomalously, the trypsin-like hK2 is found predominantly as a complex with ACT in serum, like the chymotrypsin-like PSA.[58] α-2-MG also complexes with PSA and hK2, but the proteases are completely surrounded by α-2-MG and cannot be detected by antibodies. Therefore, they are not measured in immunoassays.

Although the molecular nature of free PSA was not elucidated until several years later, free PSA represented an inactive form(s) of PSA, and for this reason was not complexed with protease inhibitors. An important part of the discovery of free PSA was its correlation with benign prostate disease. That is, free PSA is generally lower in prostate cancer than in benign prostate enlargement. This property led to the development of specific immunoassays for free PSA. Monoclonal antibodies were produced, which specifically bind to the region of PSA that is coupled to ACT in the PSA-ACT complex.[59] In this way, the free PSA antibodies will not cross react with PSA-ACT. The ratio of free PSA to total PSA is a useful clinical algorithm to increase the predictive value for positive biopsies and cancer detection. The rationale for elevated free PSA in benign disease was not revealed for several years.[60]

## The Normal Reference Range of PSA

As mentioned, in 1980 Kuriyama and colleagues[52] developed a more sensitive

method for determining the concentration of PSA in the serum, an enzyme immunoassay that contained a rabbit anti-IgG antibody to PSA. In turn, prostate tissue extracts, sera from patients with prostate cancer or other types of cancer, and sera from apparently healthy men were compared. Based on the results, the first "reference range" was formulated (0.1-1.79 ng/mL) for PSA in the serum. PSA measurements in sera from women revealed no measurable concentrations, with a lower detection limit of 0.1 ng/mL. The highest concentrations were found in the serum of patients with advanced prostate cancer. The serum PSA concentration in men with locally limited prostate cancer did not differ definitively from the PSA concentration in the serum of patients with benign prostatic hyperplasia.

The established normal range limit of 4.0 ng/mL for PSA was initially proposed in a 1986 study by Hybritech Inc., San Diego, CA, in a small population of 472 men without a history of prostate cancer. Later studies of larger and more clinically relevant populations to define the optimum cutoff value with both sensitivity and specificity for cancer detection fluctuated between 2.8 and 4.0 ng/mL. The screening study of 6630 men aged 50-74, which led to the first US Food and Drug Administration (FDA) approval for early detection, assessed the efficacy of the 4.0 cutoff and derived an upper limit of 3.9 in the 50-54 age group.[61,62] At the time, recommendation of a biopsy for men with PSA $>$ 4.0 ng/mL was considered to be an aggressive position because PSA values greater than 10 ng/mL were often viewed as the biopsy decision point.

Consequently, a value of $>$ 4.0 ng/mL became established for recommending biopsy, although it was known that men could have cancer with PSA values below 4.0 ng/mL. Numerous recent studies have shown that many cancers are missed with this cutoff,[63,64] and that earlier medical intervention may lead to improved patient outcomes.[65,66] The 2.5 ng/mL cutoff was recently recommended by the National Comprehensive Cancer Network in the United States.

## The Effect of Interassay Standardization on Reference Ranges for PSA

The clinical impact of PSA interassay variation has become a concern in the last 10 years.[67] The interpretation of PSA velocity is influencing clinical decisions, because many men present today with serial PSA determinations over several years. The study by Stephan and associates[68] shows elegantly that an increase of t-PSA of 0.5 or 0.75 ng/mL per year can be the result of a change in the assay system used rather than an actual change in PSA concentrations. Further, as recently reviewed[69] the intraindividual biological variation of t-PSA may well reach 20%. Because of such analytical and biological variations, PSA changes over time must be interpreted with great care.

*Because of analytical and biological variations, PSA changes over time must be interpreted with great care.*

Calibration of PSA assays to the World Health Organization (WHO) standard may help reduce interassay variations. Nevertheless, PSA assays incorporate different antibodies and are run on different systems. Therefore, as demonstrated by Stephan's group,[68] PSA assays are not delivering identical results even after WHO standardization. The term "harmonization" rather than "standardization" should be used to avoid the presumption that "all PSA results are the same."

Recalibration to WHO typically results in lower t-PSA concentrations than those detected by the standardization that was used to establish the original 4.0 ng/mL cutoff.[70,71] For example, a t-PSA concentration of 4 ng/mL may translate into 3.3 ng/mL after restandardization to WHO. There is a serious risk for misinterpretation of PSA if complete communication on the new cutoff is not provided after assay recalibration. Indeed, keeping a cutoff of 4 ng/mL after PSA calibration to WHO means that a man may have delayed prostate cancer detection. Up-to-date information is required from the manufacturer about adjusted cutoff values for PSA, free PSA, and percentage of free PSA when assays undergo recalibration to the WHO standard.

## PSA and Paradigm Shifts in Prostate Cancer Diagnostics

The value of using PSA as a tool for screening and early detection was not initially apparent. In fact, in the late 1980s most experts stated strongly that PSA would not be useful for this application. This was because early studies of PSA using sensitive immunoassays revealed that although PSA was elevated in prostate cancer, there was significant overlap with benign prostate disease, especially for men with enlarged prostate glands. This lack of specificity cast doubt on the clinical utility of PSA, resulting in the continued use of the established prostate marker, PAP. Lange and associates, together with Hybritech, Inc., developed a new paradigm to evaluate PSA levels following radical prostatectomy.[72]

It was hypothesized that PSA levels would drop to undetectable levels after successful surgery because the prostate gland is an essential source

of serum PSA. The use of PSA to monitor cancer relapse after curative treatment was approved by the FDA in 1986 and remains the gold standard for this clinical use. The study demonstrated that after successful prostatectomy, patients have PSA levels less than 0.1 ng/mL after the surgically released PSA is cleared from the blood. Indeed, a more recent study using an ultrasensitive PSA assay demonstrated that the "biological zero" level of PSA after surgery is below 0.008 ng/mL.[73]

These studies also demonstrate that PSA derived from the periurethral glands does not significantly enter the bloodstream. Instead, it is released into the urinary tract, invalidating urine PSA for current clinical diagnostic purposes. PSA levels above 0.1 ng/mL and continuing to rise in 2 subsequent tests over time are evidence of a biochemical relapse, which can precede a clinical relapse by months or years. The availability of FDA-approved PSA kits set the stage for the largest diagnostic paradigm shift—the use of PSA for early detection of prostate cancer. An essential part of this clinical algorithm was significant improvement in the early 1980s in the percutaneous needle biopsy guided by transrectal ultrasound (TRUS) to diagnose prostate cancer. Previously, the biopsy procedure was not very accurate, often resulted in infections, and required hospital admission. The ultrasound-guided Biopty® gun (Bard, Covington, GA) provided a valuable, easier to use tool for the urologist.[74,75]

It can be argued that without the improved biopsy procedure, PSA would not be nearly as valuable as it is today. In comparison, the cancer marker CA125 is probably more specific for ovarian cancer than PSA for prostate cancer, but the laparotomy required to diagnose ovarian cancer is an invasive surgery and greatly limits CA125 for early cancer detection. Even with an automated biopsy device such as the Biopty gun, there was a need for a more accurate selection of patients.

A multisite clinical study in the early 1990s demonstrated that sextant biopsy of patients with PSA greater than 4.0 ng/mL, with or without a suspicious digital rectal examination (DRE), was effective in patient selection for biopsy.[61] For every 100 men aged 50-75, undergoing PSA testing the first time, about 85 had PSA values less than 4.0 ng/mL. The remaining 15 men could then be evaluated by biopsy. Approximately 4-5 of these men were found to have a clinically significant cancer. This study led in 1994 to the first FDA approval for any tumor marker to aid in the early detection of cancer.

The wide use of PSA over the past 10 years has resulted in a marked change in the epidemiology of prostate cancer. The clinical stage has migrated to lower stages upon diagnosis. In 1986, fewer than 1/3 of men diagnosed with prostate cancer had organ-confined disease. Today, well over 2/3 of men diagnosed with prostate cancer have PSA values less than 10 ng/mL and potentially curable, organ-confined disease. A special clinical-stage classification, T1C, has been created for men diagnosed with prostate cancer with no other signs except an elevated PSA. The important question of whether PSA testing results in lower mortality may not be fully answered for several years, although recent data from the American Cancer Society indicate that cancer mortality has decreased since the PSA screening era began.[76] Nevertheless, it is generally accepted that the use of PSA has led to the overtreatment of many men.[77] The PSA assays in use today are not predictive in differentiating aggressive from nonaggressive cancer.

## Enhancement of PSA Tests

The use of PSA for early detection of cancer increased rapidly in the early 1990s. Today an estimated 20 million PSA tests are done per year in North America and possibly 20 million more outside of North America. With this huge number of PSA tests and subsequent large number of biopsies, a great need has emerged to increase the specificity of PSA. The discovery of the different molecular forms of PSA, free PSA, and complex PSA in 1990[78] showed the way for the next generation of immunoassays to increase PSA specificity.

Extensive research on free PSA led to specific immunoassays and a better understanding of how to utilize free PSA with total PSA to provide more predictive biopsy results. It was noted that increased levels of free PSA were associated with benign disease and a decreased probability of cancer.[79-81] Free PSA was approved by the FDA for use with total PSA for cancer detection in 1998. On the one hand, a high ratio of free PSA to total PSA (eg, $>$ 25%) greatly reduces the probability of cancer. On the other hand, a low percentage of free PSA (eg, $<$ 10%) greatly increases the probability of cancer. A limitation of free PSA is that most patients will be in the middle—the "gray on gray zone"—with a PSA between 4 and 10 and a percent of free PSA between 10% and 25%. Nevertheless, free PSA is used to aid biopsy decisions.

More recently, there have been advances in complex PSA, the other form of molecular PSA. Because the total PSA assay measures both complex and free PSA, it is expected that complex PSA will yield clinical results similar to free PSA. Most studies have demonstrated that the highly specific complex PSA assay, which is FDA-approved for giving clinical results comparable with total PSA, improves differentiation between

prostate cancer and benign biopsy results.[82] Some studies, however, have shown that complex PSA can increase predictive value for biopsy in certain PSA range intervals.[83]

Another effective way to enhance PSA specificity is to combine clinical information with a given PSA result. Independent of cancer, PSA tends to increase progressively with age.[84] The age-specific PSA reference range can be used to increase specificity for cancer. Cancer detection sensitivity for older men will be sacrificed with this method, however, resulting in many missed cancers for those over 65.[61] Nevertheless, it is generally accepted that a PSA of 3.5 for a 50-year-old man is more significant than a PSA value of 4.5 for a 70-year-old man. Reasons for this acceptance include the slow-growing nature of most prostate cancers and the overtreatment issue.

Still another effective method to increase PSA specificity is to include the size of the prostate, so-called PSA density.[85,86] Men with enlarged prostates often have elevated PSA because of benign disease. PSA density can be calculated by dividing the PSA value by the prostate volume measured by TRUS. The difficulty with this approach is that TRUS is invasive and expensive, and the prostate volumes determined at different medical centers may vary significantly. Consequently, this method is not used routinely, although biopsy decisions may be influenced even by rough size estimates by the physician. Perhaps one of the most cancer-indicative clinical features is the rate of PSA increase with time—a concept termed "PSA velocity."[87] Serum PSA increases significantly in the presence of tumor compared with benign disease alone. On the one hand, PSA velocity analysis may not be practical, because multiple PSA values must be obtained over a period of years to use this algorithm most effectively. On the other, PSA velocity may become more useful in determining the need for repeat biopsy for the increasing number of patients with negative biopsies and elevated PSA levels.[88]

## Molecular Forms of Free PSA

In spite of intensive study, the molecular nature of free PSA was unknown until the late 1990s. Free PSA was assumed to represent enzymatically inactive PSA because active PSA rapidly and irreversibly forms complexes with serum protease inhibitors. Higher percentages of inactive PSA were observed in benign prostate tissue than in normal seminal plasma.[60] The inactive PSA in the prostate tissue, more fully characterized in seminal plasma,[89] was assumed to be the origin of free PSA.

Paradoxically, the first reported free PSA form, proPSA, was found by Mikolajczyk and associates at Hybritech, Inc. to be associated with cancer and not benign disease, as would be expected for a free form of PSA.[90,91] In later studies Mikolajczyk and associates, working in conjunction with Slawin at Baylor College of Medicine, discovered another free PSA form, BPSA, which is correlated with benign disease and specifically with clinically benign prostatic hyperplasia.[92] BPSA and proPSA represent benign and cancer-associated free PSA forms and can together be considered the "yin-yang" of free PSA.[93]

The free PSA assays measure all forms of free PSA, and on average, high proportions of free PSA will be associated most often with benign prostate tissue and not cancer. In an interesting observation, 1 form of proPSA was found to be highly effective in detecting cancers in men with very high free PSA (ie, greater than 25%).[94] Specific monoclonal antibodies to BPSA and proPSA have recently been used to develop specific immunoassays for the different free PSA molecular forms.[95] ProPSA is enriched in the peripheral zone tumors, and BPSA is enriched in the transition zone, especially in enlarged glands with benign hyperplastic nodules.

A third molecular form of free PSA, inPSA (intact, nonnative PSA), has been identified in tissue and serum.[95] Unlike BPSA and proPSA, the molecular characterization of inPSA is not known. Free PSA in serum contains, on average, roughly equal proportions of the 3 molecular forms, but the relative amounts of proPSA and BPSA vary significantly in individual patients and are correlated with cancer and benign tissue. The proportion of inPSA in serum is elevated in noncancer and tends to be the inverse of proPSA. A possible explanation for the correlation of free PSA with benign tissue is the combined amounts of BPSA and inPSA.

Another specific monoclonal antibody to a free PSA form, PSA-I, detects only the nonclipped forms of free PSA.[96] Thus, the PSA-I assay would be expected to detect both proPSA and inPSA but not BPSA. Studies with PSA-I have yielded encouraging results for the detection of prostate cancer. There is no current immunoassay for inPSA, but this form can be quantified by calculation using free PSA, BPSA, and proPSA values.

*BPSA and proPSA represent benign and cancer-associated free PSA forms and can together be considered as the "yin-yang" of free PSA.*

The presence of relatively high amounts of proPSA in individual samples no doubt leads to some of the considerable overlap between cancer and noncancer with the free PSA assay. ProPSA is the natural precursor to active PSA and is rapidly cleaved to form active PSA under normal physiological conditions. In tumor cells, proPSA apparently is converted less rapidly to the active enzyme. The levels of proPSA build up in the tumor, and there is also degradation of the proPSA, which results in truncated proPSA.[91,97] The truncated forms of proPSA are resistant to conversion to active PSA enzyme. Using highly specific immunoassays for proPSA and BPSA, several studies have shown a significant increase in specificity for prostate cancer, especially at the lower PSA range, 2.5-4.0.[98,99] In addition, serum proPSA appears to provide valuable information on cancer aggressiveness.[100]

## Current Controversies in the Use of PSA in Early Detection

Recent studies have reopened the controversy regarding the appropriate level of serum PSA that should trigger a biopsy. It has been known for many years that cancer will not be found on an initial biopsy in as many as 65% of men with PSA above 4.0 ng/mL; even in early studies it was shown that men can have cancer and have normal levels of PSA. Attempts to determine the level of prostate cells in peripheral blood by reverse transcriptase polymerase chain reaction (RT-PCR) did not significantly improve cancer diagnosis or predict postoperative failure.[101] In Hybritech's initial studies in the 1980s, 19% of men with cancer had PSA values below 4.0 ng/mL.[102] Recent publication of the data from the Prostate Cancer Prevention Trial (PCPT) revealed that as many as 15% of men with a serum PSA value of less than 4.0 ng/mL have cancer and that approximately 25% of these men may have significant cancer of Gleason Score 7 or above.[64] In addition, in spite of various protocols to improve the specificity of PSA, the biopsy detection rate is only about 40% in men with PSA values from 4 to 10 ng/mL.[103] Thus, approximately 60% of these men received unnecessary biopsies.

It is clear that the measurements of new molecules from blood, urine, or other body constituents are needed to significantly reduce the cost and morbidity resulting from negative biopsies. The ideal candidate for such a marker would be exclusively expressed by prostate cancer tissue and easily sampled from the patient. An example of such a potential marker is PCA3 (formerly called DD3), a gene identified by Bussemakers and colleagues at the University of Nijmegen, the Netherlands, and Johns Hopkins University.[104] This gene, originally identified using differential display technology, is specific only to prostate cells. Furthermore, PCA3 is 60- to 100-fold overexpressed in 95% of prostate cancers as a nontranslated mRNA lacking an open reading frame.

*Recent studies have reopened the controversy regarding the appropriate level of serum PSA that should trigger a biopsy.*

Assays for PCA3 have been developed by utilizing different amplification technologies.[105-107] The tests measure the expression of the PCA3 gene in cells isolated from the urine of men suspected of having prostate cancer after receiving a digital rectal examination. These assays take advantage of the fact that PSA is expressed approximately equally by all prostate epithelial cells, whether normal or cancerous. The ratio of the expression of PCA3 as a function of the expression of the PSA gene controls for the total number of normal prostate cells in the sample.

In studies to date, the measurement of PCA3 expression as a function of PSA expression in men presenting for biopsy has yielded clinical sensitivity in the range of 50% to 75% and specificity in the range of 80% to 90%. Similar performance has been demonstrated for the detection of cancer in men presenting for a second biopsy after an initial negative result. Investigations have also shown that, unlike PSA or free PSA, the PCA3/PSA ratio is independent of prostate size. Performance characteristics such as those shown in these early research studies indicate that this marker could be a useful adjunct to PSA in determining which patients should be biopsied.

Another gene, alpha-methyl coenzyme A racemase (AMACR), has been used in this strategy for a molecular urine test. Zielie and associates demonstrated that quantification of AMACR transcripts normalized to PSA transcripts in urine sediments was highly predictive of prostate cancer.[108] The discovery of TMPRSS2:ERG gene fusions in the urine of prostate cancer patients provides a powerful new noninvasive approach to cancer detection.[109,110] Such gene translocations may be not only diagnostic but also causative for the development of prostate cancer. These are some of the more promising markers that have been proposed for use in conjunction with PSA and other mRNA markers to improve the accuracy of prostate cancer diagnostic protocols.

## Future Advances in PSA Clinical Diagnostics

Research and clinical studies over the last decade have demonstrated potential clinical applications for

PSA forms and the related prostate-localized kallikrein hK2. Individually these markers, especially proPSA, have revealed significant potential value to meet some of the difficult and currently unmet clinical needs. These include: 1) tests to determine prostate cancer aggressiveness, 2) increased specificity of PSA for biopsy decisions, especially in the 2.5-4.0 ng/mL range, and 3) predictive tests to select for repeat biopsies. Is an even greater advance on the horizon by using a combination of these markers? The technology is now available for a multiplex of analytes.[93] The simultaneous analyses of PSA, free PSA, hK2, and the molecular forms of free PSA are expected to yield a higher predictive value than any marker alone. In addition, the data could be subjected to logistic regression analysis or neural net analysis to increase the diagnostic output even further. It is entirely possible that we have not yet seen the full value of PSA, which may have some surprises for us in the near future. ■

*Portions of the text were reproduced with permission from* Touch Briefings, European Oncology Review 2005:1-3. *Semjonow A*. "Twenty Years of Prostate-specific Antigen—An Historical View."

## References


1. Ablin RJ. On the identification and characterization of prostate-specific antigen. *Hum Pathol*. 1993;24:811-812.
2. Ablin RJ. A retrospective and prospective overview of prostate-specific antigen. *J Cancer Res Clin Oncol*. 1997;123:583-594.
3. Hara M, Inoue T, Koyanagi Y. Preparation and immunoelectrophoretic assessment of antisera to human seminal plasma. *Nippon Hoigaku Zasshi*. 1966;20:365.
4. Hara M, Koyanagi Y, Inoue T, et al. Some physico-chemical characteristics of "-semino-protein," an antigenic component specific for human seminal plasma: forensic immunological study of body fluids and secretion (VII). *Nippon Hoigaku Zasshi*. 1971;25:322-324.
5. Sensabaugh GF. Isolation and characterization of a semen-specific protein from human seminal plasma: a potential new marker for semen identification. *J Forensic Sci*. 1978;23:106-115.
6. Li TS, Beling CG. Isolation and characterization of two specific antigens of human seminal plasma. *Fertil Steril*. 1973;241:134-144.
7. Wang MC, Papsidero LD, Chu TM. Prostate-specific antigen, p30, gamma-seminoprotein, and E1. *Prostate*. 1994;24:107-110.
8. Wang MC, Valenzuela LA, Murphy GP, et al. Purification of a human prostate specific antigen. *Invest Urol*. 1979;17:159-163.
9. Gutman AB, Gutman EB. An "acid" phosphatase occurring in the serum of patients with metastasizing carcinoma of prostate gland. *J Clin Invest*. 1938;17:473.
10. Armbruster D. Prostate-specific antigen: biochemistry, analytical methods, and clinical application. *Clin Chem*. 1993;39:181-195.
11. Graves HCB, Kamarei M, Stamey TA. Identity of prostate specific antigen and the semen protein P30 purified by a rapid chromatography technique. *J Urol*. 1990;144:1510-1515.
12. Lundwall A, Lilja H. Molecular cloning of human prostate specific antigen cDNA. *FEBS Lett*. 1987;214:317-322.
13. McCormack RT, Rittenhouse HG, Finlay JA, et al. Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. *Urology*. 1995;45:729-744.
14. Schaller J, Akiyama K, Tsuda R, et al. Isolation, characterization and amino-acid sequence of gamma-seminoprotein, a glycoprotein from human seminal plasma. *Eur J Biochem*. 1987; 170:111-120.
15. Sensabaugh GF. Response to: Wang MC, Papsidero LD, Chu TM. Prostate-specific antigens p30, y-seminoprotein and E1. *Prostate*. 1994;24:109.
16. Sensabaugh GF, Blake ET. Seminal plasma protein p30: simplified purification and evidence for identity: with prostate specific antigen. *J Urol*. 1990;144:1523-1526.
17. Stamey TA, Teplow DB, Graves HC. Identity of PSA purified from seminal fluid by different methods: comparison by amino acid analysis and assigned extinction coefficients. *Prostate*. 1995;27:198-203.


## Main Points

- A prostate-specific antigen (PSA) value of > 4.0 ng/mL is established for recommending biopsy. The US National Comprehensive Cancer Network has recommended 2.5 ng/mL, because many cancers are still being missed at the higher cutoff. Because PSA is not a cancer-specific marker, cancer will not be detected in 60%-75% of men upon initial biopsy.
- Men with enlarged prostates often have elevated PSA because of benign disease.
- Independent of cancer, PSA tends to increase progressively with age. The age-specific PSA reference range can increase specificity for cancer.
- Use of PSA to detect early signs of cancer relapse after treatment has allowed earlier and more effective therapies for metastatic disease.
- PSA assays are not delivering identical results, in part due to differences in PSA standardization. Tests using the more recent World Health Organization PSA standards give values 20%-25% lower than tests based on the original standardization used to establish many of the PSA cutoffs.
- Although PSA helps detect prostate cancer, PSA assays in use today are not predictive in differentiating aggressive from non-aggressive cancer. Better tests are needed to minimize overtreatment of non-life-threatening cancer.
- PSA isoforms improve cancer detection. A ratio of free PSA to total PSA > 25% greatly reduces the probability of cancer, whereas a ratio < 10% greatly increases the probability. Complexed PSA may improve cancer detection compared with total PSA. Of 2 forms of free PSA, proPSA is associated with cancer, whereas BPSA is correlated with benign disease.
- One of the more promising clinical applications for better cancer detection is PSA velocity—the rate of PSA increase with time—but effective use requires multiple PSA tests over a period of years.

18. Watt KWK, Lee PJ, M'Timkulu T, et al. Human prostate-specific antigen: structural and functional similarity with serine proteases. *Proc Natl Acad Sci USA*. 1986;83:3166-3170.
19. Sokoll LJ, Chan DW. Prostate-specific antigen: its discovery and biochemical characteristics. *Urol Clin North Am*. 1997;24:253-259.
20. Ban Y, Wang MC, Watt KWK, et al. The proteolytic activity of human prostate-specific antigen. *Biochem Biophys Res Commun*. 1984;123: 482-488.
21. Lilja H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J Clin Invest*. 1985;76:1899-1903.
22. Akiyama K, Nakamura T, Iwanaga S, et al. The chymotrypsin-like activity of human prostate-specific antigen. *FEBS Lett*. 1987;225:168-172.
23. Mikolajczyk SD, Millar LS, Kumar A, et al. Human glandular kallikrein, hK2, shows arginine-restricted specificity and forms complexes with plasma protease inhibitors. *Prostate*. 1998;34:44-50.
24. Mikolajczyk SD, Millar LS, Marker KM, et al. Ala217 is important for the catalytic function and autoactivation of prostate-specific human kallikrein 2. *Eur J Biochem*. 1997;246:440-446.
25. Lilja H. Structure and function of prostatic- and seminal vesicle-secreted proteins involved in the gelation and liquefaction of human semen. *Scand J Clin Lab Invest Suppl*. 1988;191: 13-20.
26. Deperthes D, Frenette G, Brillard-Bourdet M, et al. Potential involvement of kallikrein (hk2) in the hydrolysis of the human seminal vesicle proteins after ejaculation. *J Androl*. 1996;17:659-665.
27. Kraut H, Frey EK, Werle E. Der Nachweis eines Kreislaufhormons in der Pankreasdrüse. *Hoppe-Seylers Z Physiol Chem*. 1930;189:97-106.
28. Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev*. 1992;44:1-80.
29. Schedlich LJ, Bennetts B, Morris BJ. Primary structure of a human glandular kallikrein gene. *DNA*. 1987;6:429-437.
30. Berg T, Bradshaw RA, Carretero OA, et al. A common nomenclature for members of the tissue (glandular) kallikrein gene families. *Agents Action Suppl*. 1992;38(pt 1):19-25.
31. Rittenhouse HG, Finlay JA, Mikolajczyk SD, et al. Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. *Crit Rev Clin Lab Sci*. 1998;35:275-368.
32. Lövgren J, Airas K, Lilja H. Enzymatic action of human glandular kallikrein 2 (hK2): substrate specificity and regulation by Zn2+ and extracellular protease inhibitors. *Eur J Biochem*. 1999;262:781-789.
33. Dube JY, Deperthes D, Frenette G, et al. Human prostatic kallikrein hK2 is an active proteolytic enzyme. Poster #91 presented at: International Symposium on Biology of Prostate Growth; Mar 28, 1996, Washington, DC.
34. Becker C, Piironen T, Pettersson K, et al. Discrimination of men with prostate cancer from those with benign disease by measurements of human glandular kallikrein 2 (HK2) in serum. *J Urol*. 2000;163;311-316.
35. Deperthes D, Chapdelaine P, Tremblay RR, et al. Isolation of prostatic kallikrein hK2, also known as hGK-1, in human seminal plasma. *Biochem Biophys Acta*. 1995;1245:311-316.
36. Lovgren J, Valtonen-Andre C, Marsal K, et al. Measurement of prostate-specific antigen and human glandular kallikrein 2 in different body fluids. *J Androl*. 1999;20:348-355.
37. Takayama TK, Fujikawa K, Davie EW. Characterization of the precursor of prostate-specific antigen-activation by trypsin and by human glandular kallikrein. *J Biol Chem*. 1997;272: 21582-21588.
38. Kumar A, Mikolajczyk SD, Goel AS, et al. Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Res*. 1997;57:3111-3114.
39. Lövgren J, Rajakoski K, Karp M, et al. Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. *Biochem Biophys Res Commun*. 1997;238:549-555.
40. Bridon D, Dowell B. Structural comparison of prostate-specific antigen and human glandular kallikrein using molecular modeling. *Urology*. 1995;45:801-806.
41. Finlay JA, Day JR, Rittenhouse HG. Polyclonal and monoclonal antibodies to prostate-specific antigen can cross-react with human kallikrein 2 and human kallikrein 1. *Urology*. 1999;53:746-751.
42. Frenette G, Gervais Y, Tremblay RR, et al. Contamination of purified prostate-specific antigen preparations by kallikrein hK2. *J Urol*. 1998; 159:1375-1378.
43. Finlay JA, Day JR, Evans CL, et al. Development of a dual monoclonal antibody immunoassay for total human kallikrein 2. *Clin Chem*. 2001;47: 1218-1224.
44. Takayama TK, McMullen BA, Nelson PS, et al. Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. *Biochemistry*. 2001;40:15341-15348.
45. Fielder PJ, Rosenfeld RG, Graves HC, et al. Biochemical analysis of prostate specific antigen-proteolyzed insulin-like growth factor binding protein-3. *Growth Regul*. 1994;4:164-172.
46. Riegman PH, Vlietstra RJ, Suurmeijer L, et al. Characterization of the human kallikrein locus. *Genomics*. 1992;14:6-11.
47. Clements J. The glandular kallikrein family of enzymes: tissue specific expression and hormonal regulation. *Endocrine Rev*. 1989;10:393-419.
48. Yousef GM, Diamandis EP. The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocr Rev*. 2001;22: 184-204.
49. Clements J, Hooper J, Dong Y, et al. The expanded human kallikrein (KLK) gene family: genomic organisation, tissue-specific expression and potential functions. *Biol Chem*. 2001;382:5-14.
50. Takayama TK, Carter CA, Deng T. Activation of prostate-specific antigen precursor (pro-PSA) by prostin, a novel human prostatic serine protease identified by degenerate PCR. *Biochemistry*. 2001;40:1679-1687.
51. Papsidero LD, Wang MC, Valenzuela LA, et al. A prostate antigen in sera of prostatic cancer patients. *Cancer Res*. 1980;40:2428-2432.
52. Kuriyama M, Wang MC, Papsidero LD, et al. Quantitation of prostate-specific antigen in serum by a sensitive enzyme immunoassay. *Cancer Res*. 1980;40:4658-4662.
53. Kuriyama M, Wang MC, Lee CI, et al. Use of human prostate-specific antigen in monitoring prostate cancer. *Cancer Res*. 1981;41:3874-3876.
54. Monda JM, Barry MJ, Oesterling JE. Prostate specific antigen cannot distinguish stage T1a (A1) prostate cancer from benign prostatic hyperplasia. *J Urol*. 1994;151:1291-1295.
55. Walsh PC. Nerve sparing radical prostatectomy for early stage prostate cancer. *Semin Oncol*. 1988;15:351-358.
56. Lilja H, Christensson A, Dahlen U, et al. Prostate-specific antigen in serum occurs predominantly in complex with $\alpha_1$-antichymotrypsin. *Clin Chem*. 1991;37:1618-1625.
57. Stenman UH, Leinonen J, Alfthan H, et al. A complex between prostate-specific antigen and $\alpha_1$-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. *Cancer Res*. 1991;51:222-226.
58. Finlay JA, Evans C, Day J, et al. Detection of prostate cancer with immunoassays for free and complexed human glandular kallikrein (hK2). *J Urol*. 1997;157:147.
59. Stenman UH, Paus E, Allard WJ, et al. Summary report of the TD-3 workshop: characterization of 83 antibodies against prostate-specific antigen\par. *Tumour Biol*. 1999;20(1 suppl):1-12.
60. Chen Z, Chen H, Stamey TA. Prostate specific antigen in benign prostatic hyperplasia: purification and characterization. *J Urol*. 1997;157: 2166-2170.
61. Catalona WJ, Hudson MA, Scardino PT, et al. Selection of optimal prostate specific antigen cutoffs for early detection of prostate cancer: receiver operating characteristic curves. *J Urol*. 1994;152(6 pt 1):2037-2042.
62. Catalona WJ, Smith DS, Ratliff TL, et al. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *N Engl J Med*. 1991;324:1156-1161.
63. Punglia RS, D'Amico AV, Catalona WJ, et al. Effect of verification bias on screening for prostate cancer by measurement of prostate-specific antigen. *N Engl J Med*. 2003;349:335-342.
64. Thompson IM, Pauler DK, Goodman PJ, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or = 4.0 ng per milliliter. *N Engl J Med*. 2004;350:2239-2246.
65. Berger AP, Volgger H, Rogatsch H, et al. Screening with low PSA cutoff values results in low rates of positive surgical margins in radical prostatectomy specimens. *Prostate*. 2002;53:241-245.
66. Catalona WJ, Ramos CG, Carvalhal GF, et al. Lowering PSA cutoffs to enhance detection of curable prostate cancer. *Urology*. 2000;55:791-795.

67. Semjonow A, Brandt B. Oberpenning F, et al. Discordance of assay methods creates pitfalls for the interpretation of prostate-specific antigen values. *Prostate Suppl*. 1996;7:3-16.
68. Stephan C, Klaas M, Muller C, et al. Interchangeability of measurements of total and free prostate-specific antigen in serum with 5 frequently used assay combinations: an update. *Clin Chem*. 2006;52:59-64.
69. Soletormos G, Semjonow A, Sibley PE, et al. Biological variation of total prostate-specific antigen: a survey of published estimates and consequences for clinical practice. *Clin Chem*. 2005;51:1342-1351.
70. Link RE, Shariat SF, Nguyen CV, et al. Variation in prostate specific antigen results from 2 different assay platforms: clinical impact on 2304 patients undergoing prostate cancer screening. *J Urol*. 2004;171(6 pt 1):2234-2238.
71. Semjonow A, Oberpenning F, Weining C, et al. Do modifications of nonequimolar assays for total prostate-specific antigen improve detection of prostate cancer? *Clin Chem*. 2001;47:1472-1475.
72. Lange PH, Ercole CJ, Lightner DJ, et al. The value of serum prostate specific antigen determinations before and after radical prostatectomy. *J Urol*. 1989;141:873-879.
73. Ellis WJ, Vessella RL, Noteboom JL, et al. Early detection of recurrent prostate cancer with an ultrasensitive chemiluminescent prostate-specific antigen assay. *Urology*. 1997;50:573-579.
74. Lindgren PG. Percutaneous needle biopsy: a new technique. *Acta Radiol Diagn (Stockh)*. 1982;23;653-656.
75. Ragde H, Aldape HC, Bagley CM Jr. Ultrasound-guided prostate biopsy. Biopty gun superior to aspiration. *Urology*. 1988;32:503-506.
76. Jemal A, Siegel R, Ward E, et al. Cancer Statistics 2007. *CA Cancer J Clin*. 2007;57:43-66.
77. Draisma G, Boer R, Otto SJ, et al. Lead times and overdetection due to prostate-specific antigen screening: estimates from the European Randomized Study of Screening for Prostate Cancer. *J Natl Cancer Inst*. 2003;95:868-878.
78. Christensson A, Laurell CB, Lilja H. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur J Biochem*. 1990;94:755-763.
79. Catalona WJ, Partin AW, Slawin KM, et al. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. *JAMA*. 1998;279:1542-1547.
80. Partin AW, Catalona WJ, Southwick PC, et al. Analysis of percent free prostate-specific antigen (PSA) for prostate cancer detection: influence of total PSA, prostate volume, and age. *Urology*. 1996;48(6A suppl):55-61.
81. Woodrum DL, Brawer MK, Partin AW, et al. Interpretation of free prostate specific antigen clinical research studies for the detection of prostate cancer. *J Urol*. 1998;159:5-12.
82. Keller T, Butz H, Lein M, et al. Discordance analysis characteristics as a new method to compare the diagnostic accuracy of tests: example of complexed versus total prostate-specific antigen. *Clin Chem*. 2005;51:532-539.
83. Okihara K, Cheli CD, Partin AW, et al. Comparative analysis of complexed prostate specific antigen, free prostate specific antigen and their ratio in detecting prostate cancer. *J Urol*. 2002;167:2017-2023.
84. Oesterling JE, Jacobsen SJ, Chute CG, et al. Serum prostate-specific antigen in a community-based population of healthy men: establishment of age-specific reference ranges. *JAMA*. 1993;270:860-864.
85. Babaian RJ, Fritsche HA, Evans RB. Prostate-specific antigen and prostate gland volume: correlation and clinical application [see comments]. *J Clin Lab Anal*. 1990;4:135-137.
86. Benson MC, Whang IS, Pantuck A, et al. Prostate specific antigen density: a means of distinguishing benign prostatic hypertrophy and prostate cancer. *J Urol*. 1992;147(3 pt 2):815-816.
87. Carter HB, Pearson JD, Metter EJ, et al. Longitudinal evaluation of prostate-specific antigen levels in men with and without prostate disease. *JAMA*. 1992;267:2215-2220.
88. D'Amico AV, Chen MH, Roehl KA, et al. Preoperative PSA velocity and the risk of death from prostate cancer after radical prostatectomy. *N Engl J Med*. 2004;351:125-135.
89. Zhang WM, Leinonen J, Kalkkinen N, et al. Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid. *Clin Chem*. 1995;41:1567-1573.
90. Mikolajczyk SD, Grauer LS, Millar LS, et al. A precursor form of PSA (pPSA) is a component of the free PSA in prostate cancer serum. *Urology*. 1997;50:710-714.
91. Mikolajczyk SD, Marker KM, Millar LS, et al. A truncated precursor form of prostate-specific antigen is a more specific serum marker of prostate cancer. *Cancer Res*. 2001;61:6958-6963.
92. Mikolajczyk SD, Millar LS, Wang TJ, et al. "BPSA," a specific molecular form of free prostate-specific antigen, is found predominantly in the transition zone of patients with nodular benign prostatic hyperplasia. *Urology*. 2000;55:41-45.
93. Mikolajczyk SD, Rittenhouse HG. Pro PSA: a more cancer specific form of prostate specific antigen for the early detection of prostate cancer. *Keio J Med*. 2003;52:86-91.
94. Mikolajczyk SD, Song Y, Wong JR, et al. Are multiple markers the future of prostate cancer diagnostics? *Clin Biochem*. 2004;37:519-528.
95. Mikolajczyk SD, Marks LS, Partin AW, et al. Free prostate-specific antigen in serum is becoming more complex. *Urology*. 2002;59:797-802.
96. Nurmikko P, Vaisanen V, Piironen T, et al. Production and characterization of novel anti-prostate-specific antigen (PSA) monoclonal antibodies that do not detect internally cleaved Lys145-Lys146 inactive PSA. *Clin Chem*. 2000;46:1610-1618.
97. Peter J, Unverzagt C, Krogh TN, et al. Identification of precursor forms of prostate-specific antigen in serum of prostate cancer patients by immunosorption and mass spectrometry. *Cancer Res*. 2001;61:957-962.
98. Catalona WJ, Bartsch G, Rittenhouse HG, et al. Serum pro prostate specific antigen improves cancer detection compared to free and complexed prostate specific antigen in men with prostate specific antigen 2 to 4 ng/ml. *J Urol*. 2003;170(6 pt 1):2181-2185.
99. Sokoll LJ, Chan DW, Mikolajczyk SD, et al. Proenzyme PSA for the early detection of prostate cancer in the 2.5-4.0 ng/ml total PSA range: preliminary analysis. *Urol*. 2003;61:274-276.
100. Catalona WJ, Bartsch G, Rittenhouse HG, et al. Serum pro-prostate specific antigen preferentially detects aggressive prostate cancers in men with 2 to 4 ng/ml prostate specific antigen. *J Urol*. 2004;171(6 pt 1):2239-2244.
101. Ellis WJ, Vessella RL, Corey E, et al. The value of a reverse transcriptase polymerase chain reaction assay in preoperative staging and followup of patients with prostate cancer. *J Urol*. 1998;159:1134-1138.
102. Myrtle JF, Ivor LP. Measurement of prostate-specific antigen (PSA) in the serum by a two-site immunometric method (Hybritech Tandem-R/Tandem-E PSA). In Catalona WJ, Coffey DS, Karr JP, eds. *Clinical Aspects of Prostate Cancer: Assessment of New Diagnostic and Management Procedures*. New York, Elsevier; 1989:161-171.
103. Emiliozzi P, Longhi S, Scarpone P, et al. The value of a single biopsy with 12 transperineal cores for detecting prostate cancer in patients with elevated prostate specific antigen. *J Urol*. 2001;166:845-850.
104. Bussemakers MJ, van Bokhoven A, Verhaegh GW, et al. DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. *Cancer Res*. 1999;59:5975-5979.
105. Fradet Y, Saad F, Aprikian A, et al. uPM3, a new molecular urine test for the detection of prostate cancer. *Urology*. 2004;64:311-315.
106. Groskopf J, Aubin SM, Deras IL, et al. APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer. *Clin Chem*. 2006;52:1089-1095.
107. Hessels D, Klein Gunnewiek JM, van Oort I, et al. DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer. *Eur Urol*. 2003;44:8-15.
108. Zielie PJ, Mobley JA, Ebb RG, et al. A novel diagnostic test for prostate cancer emerges from the determination of alpha-methylacyl-coenzyme a racemase in prostatic secretions. *J Urol*. 2004;172:1130-1133.
109. Laxman B, Tomlins SA, Mehra R, et al. Noninvasive detection of TMPRSS2: ERG fusion transcripts in the urine of men with prostate cancer. *Neoplasia*. 2006;8:885-888.
110. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science*. 2005;310:644-648.